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REVIEW ARTICLE

Micrometastasis in Colorectal Carcinoma: A Review

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Lymph node metastasis is the most important predictor of prognosis, after surgery, in colorectal carcinoma. The term ''micrometastasis'' has evolved from a morphological definition to one that is used with molecular-based techniques. We review the literature to evaluate the significance of detecting micrometastases in colorectal carcinoma, either by morphological or molecular techniques, and address technical difficulties encountered with both. Routine use of immunohistochemistry is not recommended as most studies show little change in staging or prognosis. Radioimmunoguided surgery may prove beneficial, but problems of false positives in benign diseases need to be addressed. Immunohistochemical detection of micrometastatic deposits in bone marrow aspirates holds the most promise for clinical practice. Molecular techniques are more sensitive than immunohistochemistry, but prognostic value needs to be determined. Molecular diagnostics can also determine genetic alterations and mutations that should improve our understanding of metastatic colon cancer and staging accuracy.

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INTRODUCTION

The identification and detection of metastases in regional lymph nodes is routine practice for surgical pathologists and one of the most significant prognostic factors of the staging process. Pathologists and surgeons have long sought techniques that either improve lymph node yield or tumor cell detection, thereby improving the accuracy of staging. Today's concept of micrometastasis can be traced back to the term ''obscure metastases,'' originally associated with ways of improving and standardizing the retrieval and processing of lymph nodes removed with neoplastic tissue [1]. The notion of a metastasis being ''obscure'' or ''occult'' was first thoroughly studied in breast cancer patients and implied that improved methods were needed to detect metastatic breast carcinoma cells in lymph nodes [1,2]. Even when routine hematoxylin and eosin (H&E) staining was the only method available, step and serial sectioning would identify ''occult'' metastases in 22% of breast cancer patients [2].

The terms ''micrometastasis'' and ''macrometastasis'' were originally defined as metastatic deposits of breast carcinoma cells measuring less than or more than 2 mm, respectively [3]. As the word evolved, micrometastasis retained a morphological connotation and referred to a

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small focus of metastatic cancer often not identified because of the way in which lymph nodes were sampled [4]. Many studies were concerned with improving the sensitivity of these methods by the immunohistochemical detection of tumor cells, either in the lymph nodes or bone marrow. With the emergence of molecular diagnostics, the term ''micrometastasis'' has remained but is rarely defined, suggesting retention of its morphological origin. The use of molecular-based assays for detecting micrometastases can provide a far more sensitive technique than routine H&E histology or immunohistochemistry. Moreover, analyzing certain markers by molecular assays can detect mutations not assessable by routine H&E or immunohistochemistry as well as provide more information concerning the pathogenesis of a tumor being studied. Thus the concept of micrometastasis is being extended to mutations that may or may not improve the accuracy of staging.

Our aim is to review the significance of micrometastasis in colorectal carcinoma identified by either morphological or molecular techniques. We want to determine if the increased detection of micrometastases is associated with prognostic value, particularly in early stage node-negative patients where it may influence the use of adjuvant chemotherapy, and whether molecular techniques improve the accuracy of their identification or provide unrelated information.

MORPHOLOGICAL IDENTIFICATION OF MICROMETASTASES

Lymph Node Micrometastases

Clearance technique. In colorectal carcinoma, lymph node metastasis is the most important predictor of prognosis after surgery. As in breast carcinoma, the importance of lymph node metastases in determining prognosis first concentrated on developing methods of improving their gross identification in resected specimens, thereby improving their yield and increasing the likelihood of finding more metastatic deposits in lymph nodes. Cawthorn and colleagues [5] developed a xylene alcohol clearance technique that involved formalin fixation and repeated washes in ethanol followed by clearance in xylene. A significantly higher yield of total and metastatic lymph nodes was obtained compared to other centers, but the incidence of Dukes' C tumors was not significantly changed [5]. Jass and associates [6] did not show significant improvement in the number of recovered lymph nodes.

Immunohistochemical technique. Several studies have employed immunohistochemistry to detect micrometastases using monoclonal antibodies (MAbs) directed against carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), or cytokeratin (CK). O'Brien's group [7] performed immunoperoxidase and immunofluorescence staining on paraffin-embedded and frozen tissue sections, respectively. They applied a MAb to CEA on sections of primary tumors, lymph nodes, surgical margins, polyps, and normal mucosa [7]. All 150 lymph nodes, originally diagnosed as negative by routine histology, remained negative when examined by immunoperoxidase staining [7]. They concluded that, although lymph node micrometastases may be more easily detected by immunohistochemistry with CEA, screening of H&E-stained sections by a competent pathologist appeared to be equally sensitive [7]. Davidson and colleagues [8] examined regional lymph nodes from 48 colon cancer specimens by conventional H&E staining and with MAbs directed against CEA and EMA. Of 200 lymph nodes reported as free of tumor deposits by H&E, a micrometastasis was found in only one lymph node, which altered the staging from Dukes' B to Dukes' C [8]. These tumor cells, however, had been overlooked, and on re-examination were visible on the original H&E [8]. The overall results did not suggest a useful role for routine immunohistochemistry of lymph node sections in conjunction with H&E [8]. Cutait and colleagues [9] reviewed 46 cases of colorectal adenocarcinoma in patients originally reported as not having lymph node metastases. They identified tumor cells in 22 lymph nodes from 12 patients based on immunohistochemical detection of CEA and CK [9]. When these patients were restaged, however, into those with lymph node metastases and those without, there was no statistical difference in 5-year survival [9]. Jeffers and associates [10] detected micrometastases in 19 cases (25%) by immunohistochemical staining for CK AE1:AE3. The presence of micrometastases was not correlated to age, gender, tumor site, size, and differentiation [10]. Five- and 10-year survival rates showed no significant difference between the patients with or without micrometastases [10]. They therefore concluded that detecting nodal micrometastases by immunohistochemistry did not warrant reassignment to a more advanced stage [10]. A recent Swedish study [11] reached similar conclusions in their analysis of 100 Dukes' B patients. By re-examining regional lymph nodes using MAbs against CK, micrometastases were detected in 39% of the patients [11]. The outcome of these patients, however, was not significantly different from those patients having negative lymph nodes [11]. Thus many studies have shown that little additional prognostic information is obtained with immunohistochemistry.

In contrast, a study by Greenson's group [12] provided data that micrometastases do influence prognosis. After reviewing 568 pericolic lymph nodes removed from 50 patients with Dukes' B colorectal carcinoma, immunohistochemical staining was performed using antibodies to cytokeratin (AE1/AE3) and a second-generation MAb, CC49, directed against tumor-associated glycoprotein-72 (TAG-72) [12]. CK staining revealed occult metastases in 5.8% of the nodes examined, upstaging 28 patients.

CK-positive cells within lymph nodes correlated with a significantly poorer prognosis, but there was no significant difference in survival between the CC49-positive and CC49-negative groups [12]. Consequently, they recommended CK staining of pericolic lymph nodes in patients with Dukes' B colorectal carcinoma [12].

Combined clearance and immunohistochemical techniques. By combining the xylene clearance method with immunohistochemical staining, Haboubi and colleagues [13] showed an 88% increase in recovery of lymph nodes. After immunostaining with a monoclonal anticytokeratin antibody (CAM 5.2), Dukes' staging changed in 12 out of 41 cases, with 55% of Dukes' B becoming Dukes' C [13]. They strongly recommended prospective survival studies for assessing prognostic significance [13]. Nicholson and colleagues [14] reached the opposite conclusion. They examined 542 lymph nodes which had undergone xylene clearance for removal of pericolic fat and were negative by routine sectioning [14]. The sections were either immunohistochemically stained, using CAM 5.2, or serial sectioned three times at 75 μm intervals [14]. None of the 542 nodes underwent a change in status from negative to positive, and they concluded that immunohistochemistry and leveling of nodes do not increase the accuracy of pathological staging above that of xylene clearance [14].

Combined surgery and immunohistochemical technique. A new technique has been developed to detect metastasis at the time of surgery utilizing ^{125}I-radiolabeled CC49 antibody to TAG-72 [15]. Referred to as RIGS for radioimmunoguided surgery, colorectal carcinoma patients are injected with the radiolabeled antibody 3 weeks prior to surgery and then undergo intraoperative surveillance using a hand-held, gamma-detecting probe to search for metastatic disease that would normally not be resected [15]. Since some RIGS-positive areas are negative by clinical examination or routine histology, a study by Cote and researchers from Greenson's group [15] was performed to determine if these areas could be attributed to occult metastases. Fifty-seven lymph nodes were resected from nine patients newly diagnosed with colorectal carcinoma and seven patients with recurrent disease [15]. Paraffin sections were cut at 5 levels, 50 μm apart, examined by H&E, and immunohistochemically stained with a cocktail of MAbs to CK. H&E staining revealed tumor in 17 lymph nodes, whereas 40 were considered negative [15]. Thirty-nine of the 57 nodes were RIGS positive, 14 of which were also H&E positive [15]. Of the 25 RIGS-positive, H&E-negative lymph nodes, 10 (40%) demonstrated occult metastases after serial sectioning and immunohistochemical analysis [15]. Therefore, a total of 27 lymph nodes contained metastatic carcinoma, with 17 of 27 (63%) detected by routine histology and 24 of 27 (89%) detected by the probe [15]. The RIGS procedure was found to be more sensitive than clinical or histological examination. Moreover, a negative lymph node by routine histology but positive by RIGS was associated with the presence of occult lymph node metastases identified by immunohistochemical techniques [15]. These findings may have significant prognostic value as RIGS-positive, H&E-negative patients may have a worse prognosis than RIGS-negative, H&E-negative patients [15].

The RIGS method may not predict prognosis because of its high rate of false-positive results. Thirteen of the 39 (34%) RIGS-positive lymph nodes had no evidence of tumor even after serial sectioning and immunohistochemical analysis [15]. This immunoreactivity may be due to drainage of cellular breakdown products from the primary tumor, suggesting that the RIGS procedure may be useful in identifying new sites of lymphatic drainage of colorectal carcinoma [15]. An alternative explanation considers the TAG-72 antigen. A first-generation MAb, B72.3, directed against TAG-72, has been repeatedly immunoreactive in benign diseases [16–21]. Loy and Haege used B72.3 and avidin-biotin immunohistochemistry on paraffin-embedded sections to study benign intra-abdominal lymph nodes and determine the specificity of TAG-72 as a marker for metastatic colonic adenocarcinoma [22]. They retrospectively evaluated 276 benign lymph nodes taken from 35 cases of colonic adenocarcinoma and 33 cases of benign gastrointestinal disorders [22]. They found B72.3 immunoreactivity in germinal centers of 49% of the benign lymph nodes associated with adenocarcinoma and 12% associated with benign disease [22]. They concluded that immunoreactivity for TAG-72 may represent the labeling of free TAG-72 antigen in benign lymph nodes draining gastrointestinal lesions that may be unrelated to metastatic disease [22]. These results are supported by Greenson's study [12]. TAG-72 immunostaining was demonstrated in germinal centers of histologically negative lymph nodes from 38 of 50 (76%) cases of colonic carcinoma using CC49 [12].

Bone Marrow Micrometastases

The immunohistochemical detection of isolated tumor cells in the bone marrow from patients with carcinomas that rarely spread to bone (e.g., colon) has been examined. This organ is easy to access and native mesenchymal cells can be immunocytochemically distinguished from disseminated epithelial cells. Schlimok's group [23] originally used a panel of MAbs against CK and 17-1A epithelial antigen to immunocytochemically detect tumor cells in the bone marrow of 57 colorectal and 155 breast cancer patients. Briefly, the assay entails 1 or 2 aspirates from the upper iliac crest yielding greater than 1.5×10^5 mononuclear bone marrow cells per aspirate, a broad-spectrum cytokeratin monoclonal antibody, the alkaline phosphatase/anti-alkaline phosphatase method, and analysis by independent observers [23,24]. Alkaline

phosphatase was used rather than horseradish peroxidase because of the high level of endogenous peroxidase found in the bone marrow. A positive reaction with MAb CK2 was seen in 12 of 57 patients [23]. There was no correlation between the incidence of CK-positive cells and presence of distant metastasis. CK-positive cells, however, were found in a significantly higher frequency in Dukes' C patients [23]. A second study by this group showed that 42 of 156 colorectal carcinoma patients presented with CK-positive cells in the bone marrow at the time of surgery and had a significantly higher relapse rate [25]. A further study by this group examined 88 patients with colorectal carcinoma [26]. Twenty-eight (32%) of the patients were smear positive [26]. Prognostic value was determined in a follow-up study with a median observation time of 35 months, and patients with tumor cells in their bone marrow aspirates showed a significantly shorter disease-free survival [26]. A Cox regression model multivariate analysis showed that tumor cells in the bone marrow were an independent, significant determinant of relapse [26]. A second group, using the same technique, looked at seven patients who underwent radical resection for colorectal carcinoma and had no evidence of metastasis [27]. They performed bone marrow aspirates 2-6 weeks after surgery and found tumor cells in 5 of 7 patients [27]. Moreover, of these seven patients, two had a very high number of tumor cells [27]. One died 11 months after surgery and the second had a local relapse 21 months after surgery [27]. They also looked at 12 patients with metastatic disease and found two patients with tumor cells in the bone marrow [27].

Pantel and colleagues [28] have attempted to phenotype CK-positive tumor cells in the bone marrow of colorectal and breast carcinoma patients using an immunohistochemical double-labeling method. They applied a MAb to CK and a second target antigen such as Ki-67, p120, the tyrosine kinase receptor erb-B2, or the major histocompatibility complex (MHC) class I antigens [28]. They found Ki-67 or p120 to be positive in only 7 of 38 (18%) patients, regardless of their tumor type, suggesting that the tumor cells were resting in G0 or early G1 phase of the cell cycle [28]. This is consistent with the idea of tumor cell dormancy in which disseminated tumor cells can hibernate for many years until metastatic disease becomes clinically apparent [28]. In 8 of 28 (29%) colorectal carcinoma and 48 of 71 (68%) breast cancer patients, erb-B2 was positive, possibly indicating that colon cancer cells in bone marrow inherit less of a proliferative potential than breast cancer cells [28]. Moreover, 12 of 17 (71%) of colorectal carcinoma patients, as opposed to 9 of 26 (35%) of breast cancer patients, showed positivity for MHC class I antigens, suggesting that colon cancer cells cannot escape T-cell mediated cytotoxicity as easily as breast cancer cells [28].

Juhl and colleagues [29] used immunocytochemical methods to compare cytological samples from peritoneal lavages to bone marrow aspirates from gastric, colorectal, and pancreatic cancer patients. They used MAbs against tumor-associated antigens (CEA, CA 19-9, 17-1-A, C-54-0, and Ra96) and compared them to MAbs against cytokeratins (KL-1) [29]. The rate of micrometastatic cell detection in the peritoneum and bone marrow compartments was similar: 27% and 29% of colorectal carcinoma patients, respectively. The combined evaluation of both compartments, however, significantly increased the detection rate to 40% [29]. The prognostic value of these methods needs to be determined with follow-up studies.

Limitations of Immunohistochemical Methods

Although immunohistochemistry can be an important supportive tool, problems with this methodology continue to surface. The problems of standardization and antibody selection, for example, persist even though a collaborative document from the Division of Clinical Laboratory Devices of the FDA and the Biological Stain Commission Steering Committee attempted to eliminate them [30]. Antigen retrieval methods have received significant attention and have been summarized best by ''Gown's Law'': with sufficient manipulation, any antibody can be made to stain any tissue [31]. A recent editorial by Swanson [31] pointed out that retrieval methods have not led to a common method but to a wide array of disparate protocols. He asked whether the future of immunohistochemistry holds a unique retrieval method for each antibody in the laboratory, and by applying Gown's accepted acronym for antigen retrieval (heat-induced epitope retrieval, or HIER), stated that we presently approach a level of confusion in immunohistochemistry that might best be described as ''HIER*anarchy*'' [31]. As none of the immunohistochemical studies mentioned in this review consider antigen retrieval, all are open to criticism regarding their validity.

Another problem with immunohistochemistry concerns the interpretation of results. At first, detecting immunohistochemical positivity appears deceptively to be a low power exercise. The art, however, involves distinguishing between artifact, background, cross-reactivity, and actual positive staining. One needs to know if the antibody in question stains primarily the nucleus or cytoplasm and whether an unexpected staining pattern can be interpreted as a negative result, or is attributed to the antibody or method used. A fourth problem deals with antibody selection. Two monoclonal antibodies directed against a particular gene product may not recognize the same epitope, and each antibody can have different cross-reactivity patterns.

Summary

In summary, routine use of immunohistochemistry, in conjunction with H&E for detecting micrometastases in

TABLE I. Morphologic Identification of Micrometastases in Lymph Nodes

Study	No. of patients	Technique	Increased detection	% of patients upstaged	Prognostic significance
Cawthorn et al. [5]	272	XC[a]	Yes	None	NA[d]
Jass et al. [6]	20	XC	No	None	None
O'Brien et al. [7]	90	IH[b]	No	None	None
Davidson et al. [8]	47	IH	Yes	2	None
Cutait et al. [9]	46	IH	Yes	26	None
Jeffers et al. [10]	77	IH	Yes	None	None
Adell et al. [11]	100	IH	Yes	39	None
Greenson et al. [12]	50	IH	Yes	28	Yes
Haboubi et al. [13]	47	XC + IH	Yes	29	NA
Nicholson et al. [14]	33	XC + IH	No	None	None
Cote et al. [15]	16	IH + surgery[c]	Yes	NA	Yes

[a]XC, xylene clearance.
[b]IH, immunohistochemistry.
[c]Radioimmunoguided surgery (RIGS).
[d]NA, not available.

lymph nodes, is not recommended, as most studies show little change in staging or prognosis (see Table I). Greenson's work [12], using CK to stain pericolic lymph nodes in patients with Dukes' B disease, has yet to be confirmed by other groups but could be beneficial. It is unclear whether Haboubi's improvement in detection can be attributed to improved lymph node detection or histochemical technique and whether it influences prognosis [13]. The use of RIGS is a promising technique for identifying clinically significant micrometastases [15]. The problems of false positives in benign diseases and a 3-week waiting period between injection and surgery, however, must be addressed before RIGS has broad clinical application in colon cancer [22]. The approach with the most promise for immediate change in clinical practice is the immunohistochemical detection of micrometastatic deposits in preoperative or perioperative bone marrow aspirates from colorectal carcinoma patients (see Table II). Prognostic significance needs to be confirmed in the United States since all of the reported data are from Europe, emerging essentially from a select group of researchers [23–29].

TABLE II. Immunohistochemical Identification of Micrometastases in Bone Marrow

Study	No. of patients[a]	Increased detection	% of patients upstaged[b]	Prognostic significance
Schlimok et al. [23]	57	Yes	ND[c]	Yes
Schlimok et al. [25]	156	Yes	ND	Yes
Lindemann et al. [26]	88	Yes	ND	Yes
Silly et al. [27]	7	Yes	71	ND
Pantel et al. [28]	45	Yes	ND	ND
Juhl et al. [29]	67	Yes	ND	ND

[a]Number reflects only colorectal carcinoma patients.
[b]Refers to patients who originally had no known metastatic disease and found to have micrometastases in the bone marrow.
[c]ND, not determined.

MOLECULAR IDENTIFICATION OF MICROMETASTASES

Polymerase Chain Reaction

The development of polymerase chain reaction (PCR)-based assays has provided an extremely sensitive diagnostic tool [32]. With its introduction as a routine methodology in the clinical laboratory, several centers have considered using it in molecular-based assays for micrometastases detection. By isolating RNA and adding the enzyme reverse transcriptase (RT) to such an assay (RT-PCR), gene expression can be detected as opposed to the presence or absence of a tumor marker. Smith and colleagues [33], for instance, used RT-PCR to amplify the gene for tyrosinase, specific for melanocytes, and were able to detect a single melanoma cell in 2 ml of blood. Mattano's group employed a very sensitive assay that detected rare circulating neuroblastoma cells by using RT-PCR amplification of the neuroendocrine protein gene product 9.5 (PGP 9.5) as a specific tumor marker for neuroblasts [34]. Moreno and colleagues [35] used prostate-specific antigen mRNA as a marker to detect circulating tumor cells in the peripheral blood. All of these studies viewed RT-PCR as a new and improved way to increase sensitivity over serial sectioning and immunohistochemistry. RT-PCR has become the most common PCR-based method currently used for detecting micrometastases in lymph nodes of colorectal carcinoma patients.

Lymph Node Micrometastases

Mori and associates [36] used an RT-PCR-based technique to study the expression of CEA mRNA in lymph nodes of patients with gastrointestinal or breast carcinoma. A total of 117 lymph nodes were obtained from three patients with rectal adenocarcinoma, as well as six

patients with gastric, two patients with esophageal, and two patients with breast carcinoma [36]. Thirty of 117 lymph nodes were positive for metastases by routine histology, but this number increased to 77 when RT-PCR was used [36]. In rectal carcinoma, 4 of 23 lymph nodes were positive by routine H&E, but 19 of 23 lymph nodes were positive by RT-PCR. They concluded that this method could save time and was cost effective and superior to routine histological examination [36]. Although larger studies need to be conducted, this method could identify high risk patients with subclinical lymph node metastases [36]. Successful RT-PCR, however, is contingent upon the gene in question being transcriptionally active in malignant cells and not in normal cells of the tissue being examined. Proliferating nonneoplastic colonic tissue can show scattered positive cells, but it is distinctly unusual to find CEA-positive cells in benign lymph nodes [37].

A second PCR-based method utilized the detection of altered DNA in the form of mutations or rearrangements. Hayashi and colleagues [38] addressed whether this technique, referred to as mutant allele-specific amplification (MASA), would allow the genetic diagnosis of occult metastases if the molecular alteration in the primary tumor could be identified. They looked for mutations in K-*ras* (codons 12, 13, and 61) and *p53* (exons 5–8) in 22 cancer specimens [38]. After detection, they used MASA to analyze regional lymph nodes for the presence or absence of cells containing the same mutations [38]. This technique proved to be more sensitive than conventional histology for detecting micrometastatic colorectal cancer cells [38]. More importantly, the presence of cancer cells in histologically negative lymph nodes could represent early dissemination and may identify Dukes' A or B patients who will develop metastatic disease [38].

In a second study by Hayashi and colleagues [39], 120 patients with colorectal carcinoma and negative lymph nodes at time of surgery were screened for the same mutations as in their earlier study and MASA identified mutations in 71 tumors [39]. Of 450 lymph nodes removed from patients with recurrent disease and originally diagnosed as negative, 184 (40.9%) contained cells with genetic alterations found in primary tumors. Of 581 lymph nodes from patients without recurrence, including Dukes' A patients, only 79 (13.6%) were diagnosed as containing metastasis by MASA [39]. All patients with distant metastases or local recurrence were lymph node positive by MASA but, more importantly, all Dukes' B patients with recurrence had positive lymph nodes by MASA [39]. Those Dukes' B patients whose lymph nodes were negative by MASA had no recurrence [39]. An unresolved problem was that 23% (10 of 44) of Dukes' A patients were MASA positive but free of recurrent disease [39]. Nevertheless, this study is the best evidence to date that molecular diagnostics may help determine who should receive postoperative adjuvant chemotherapy.

Peripheral Blood and Bone Marrow Micrometastases

Hardingham's group [40] developed a PCR-based assay for detecting circulating tumor cells. They originally used immunomagnetic beads labeled with Ber-EP4, an epithelium-specific MAb, to enrich for epithelial cells, and colorectal carcinoma cell lines as the target cells [40]. In a subsequent study, perioperative peripheral blood samples from 27 patients, whose tumors were positive for a mutation in K-*ras* codon 12, were examined [41]. PCR was performed on the isolated epithelial cell/bead aggregates [41]. The amplified products were digested with restriction enzyme *Bst*N1, run on a 10% polyacrylamide gel, and were detected using ethidium bromide or silver staining [41]. The sensitivity of the assay was 1 tumor cell in 10^6 white blood cells or 10 tumor cells/1 ml of whole blood [41]. Circulating K-*ras* mutant cells were detected in 9 of 27 patients, 7 of whom died from recurrent or metastatic disease [41]. Mutant cells were not detected in 18 patients, and 16 of 18 have remained disease free, indicating that K-*ras* mutant cells in blood were associated with a significant decrease in disease-free survival [41].

Soeth and colleagues [42] used nested RT-PCR to detect CK-20 mRNA in bone marrow specimens from 57 patients with colorectal carcinoma. They first amplified a region from exons 1 and 5, using two sets of primers for CK-20, and used this product for further amplification with primers from exons 1 and 4 [42]. They found that 20 of 57 (35%) of the bone marrow samples tested positive for CK-20 with a breakdown by stage as follows: none in stage I, 24% in stage II, 31% in stage III, and 71% in stage IV [42]. Follow-up studies need to be performed to determine prognosis. It should be noted that another group has detected CK-20 using a nonnested RT-PCR approach that is quick, easy to use, avoids the contamination problem that can easily occur with nested PCR, and hence would be clinically more relevant [43].

Limitations

The primary disadvantage of PCR-based assays is the potential contamination from previously amplified products. This problem is usually alleviated by adhering to meticulous technique and maintaining separate areas for nucleic acid extraction, reagent preparation, and nucleic acid amplification. Moreover, several technical factors could potentially limit the use of RT-PCR. First, since RNA is usually not extractable from formalin-fixed tissue, retrospective studies cannot be performed on paraffin sections. The tissue needs to be snap-frozen or placed immediately in guanidine isothiocyanate. Second, RNA preparation, although routine in molecular pathology

laboratories, requires meticulous technique to protect from RNase activity. Third, one needs to control for contamination of genomic DNA by carefully selecting primers and utilizing proper controls. Fourth, as with PCR in general, cross-contamination between specimens must be avoided by utilizing separate areas for nucleic acid extraction, reagent preparation, and nucleic acid amplification.

In attempting to improve the sensitivity and specificity of micrometastasis detection, Neumaier and colleagues [44] planned to design a PCR-based assay for CK-18 and CEA but serendipitously encountered another problem with RT-PCR—the presence of a CK-18 pseudogene. Defined as a DNA sequence that shows a high degree of sequence homology to a nonallelic functional gene, but is itself nonfunctional, pseudogenes are stable components of the genome that can be transcribed easily because they often lack introns [45]. Consequently, the high sensitivity of a PCR-based assay could amplify the pseudogene generating false positive results. This discovery of a pseudogene in CK-18 was supported by the following results: specific amplification was readily obtained from healthy bone marrow donors, reverse transcriptase was not required for CK-18 mRNA, and the pseudogene was not abolished by RNase treatment [44].

Finally, because lymph nodes are antigen processing centers, a degenerating tumor may produce fragments of DNA that are transported to a lymph node, as encountered with TAG-72 in the RIGS study [15,22]. These fragments may contain a target nucleic acid sequence that could be misinterpreted as presence of neoplastic cells and also give false positive results [46].

Advantages Over Immunohistochemistry

Standardization of assays between laboratories is not routinely encountered if a laboratory has established credibility through passing of inspections, proficiency testing, and publications. Moreover, molecular-based methods are more readily reproducible since the key component to a PCR-based assay is knowing the gene in question (i.e., the targeted nucleic acid region to amplify) and the proper primer design. Once a group develops a reproducible assay, there are usually no major discrepancies since a given primer sequence results in an amplified product of a particular base pair length detectable on gel electrophoresis. The specificity of an amplified product is frequently confirmed by hybridization with a specific probe.

The notion that successful RT-PCR is contingent upon whether a gene in question is transcriptionally active applies to genes such as K-*ras* or *p53* whose mutations commonly occur in several exons. Molecular-based assays have an advantage over immunohistochemistry because methods such as MASA or the Non-Isotopic RNase Cleavage Assay (NIRCA™; Ambion, Austin, TX) can compensate for a point mutation occurring on one of several loci by designing appropriate primers or sets of primers to cover various exons [47]. Immunohistochemistry would require the application of several MAbs designed so that the epitopes would specifically match the locations in question, an approach that would be costly and time-consuming.

DISCUSSION

The term ''micrometastasis'' has evolved from an association with retrieval and processing of lymph nodes to immunohistochemical or molecular methods that increase the sensitivity of detection. A central unresolved issue is whether increasing the yield of lymph nodes with micrometastases is clinically important. A recent study from William Beaumont Hospital [48] has demonstrated that, although all lymph nodes should ideally be recovered from a colon or rectum specimen, in practice at least 17 should be retrieved. In colorectal carcinoma, regardless of the approach used, the consensus is that prognosis is directly related to the number of involved lymph nodes [49]. As seen from our review, however, this conclusion may not be entirely accurate.

Prognostic Significance and Future Developments

The prognostic significance of detecting nodal micrometastases in colorectal carcinoma patients remains unresolved. Consequently, whether their detection could ultimately benefit the population of Dukes' A and B patients who have recurrent disease and have a decreased 5-year survival needs to be determined. Routine immunohistochemical examination of lymph nodes is not recommended since most studies show minimal or no prognostic significance [7–11]. The combined technique of fat clearance for lymph node detection followed by immunohistochemical examination may increase lymph node yield, but its prognostic significance has not been demonstrated [13,14]. RIGS may be beneficial, but its problems, especially of false positives in benign disease, need to be resolved before widespread clinical use is advisable [15]. Immunohistochemical detection of bone marrow aspirates may prove to be the most clinically significant [23–29]. Since PCR-based assays are the most sensitive available in the diagnostic laboratory, often making the detection of 1 cell in 10^6 possible, their use has increased the detection of micrometastatic deposits in lymph nodes [36,38]. Although larger studies need to be conducted to determine prognostic significance, the PCR-based MASA method may provide a molecular approach for risk stratifying Dukes' B patients [39]. PCR has been used to detect K-*ras* mutant cells in peripheral blood and showed a correlation between the presence of tumor cells and shortened disease-free survival [41]. RT-PCR has improved the detection rate of micrometastatic deposits

in bone marrow aspirates, but prognostic significance has yet to be established [42].

We are just beginning to see the potential that molecular diagnostics provides in studying alterations or mutations in a tumor. In colon cancer, the results of these studies may provide a new means of staging tumors and determining which node-negative colon cancer patients should receive adjuvant therapy. Moreover, the results could stratify these patients based upon who will benefit from a nodal dissection, thereby eliminating unnecessary surgery and the potential associated morbidity. The sentinel lymphadenectomy, for instance, may become the standard procedure for staging breast cancer in the future [50]. Since the first (''sentinel'') axillary lymph node is the most likely site of axillary metastasis, and histopathologic examination correlates well with an examination of the entire axilla, future surgeons may only have to remove one node (or a few) for molecular analysis and stage a patient accordingly. In addition, one group [4] recently used molecular techniques in assessing surgical margins in squamous cell carcinoma of the head and neck.

In conclusion, metastatic colon cancer is currently viewed as a progression of neoplastic disease, involving a series of unknown genetic alterations [51]. Molecular analysis has the potential to offer a better understanding of these alterations and thereby direct us to appropriate markers for the development of a clinically useful, molecular-based assay for the detection of micrometastases. Finally, since molecular techniques detect mutations or alterations in a tumor's DNA, none of which could be evaluated by histological or immunohistological staining, we propose the abandonment of the term ''micrometastasis'' when used in the context of molecular diagnostics. In its place we suggest one of the following: genetic alterations suggestive of potential metastatic disease, or molecular analysis of metastatic disease.

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